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ARTICLE

Caenorhabditis elegans as an *in vivo* model to assess FUCOIDAN bioactivity preventing *Helicobacter pylori* infection

Received 00th January 20xx,
Accepted 00th January 20xx

DOI: 10.1039/x0xx00000x

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Currently, *Helicobacter pylori* is a unique biological carcinogenic agent. The search for antimicrobial alternatives to antibiotics against this pathogen has been categorized as a priority due to the drastic failure associated with current applied antibiotic therapy. The present study assessed the bioactive antimicrobial capability of fucoidan ("Generally Recognized as Safe" approval – European Commission December 2017)[1] from different species of *Phaeophyceae* algae (*Fucus vesiculosus, Undaria pinnatifida, Macrocystis pyrifera*) against *H. pylori*. All studied fucoidans showed bacteriostatic and bactericidal effects at the studied concentrations [5-100] µg/ml, and exposure times [0-7] days. The most effective anti-*H. pylori* fucoidan was validated in *Caenorhabditis elegans* as an *in vivo* model. *C. elegans* feed was supplemented with *Undaria pinnatifida* [0-100] µg/ml fucoidan, resulting in a significant improvement in lifespan, lowered *H. pylori* concentration in digestive tract, and increased egg-laying pattern. New research lines proposing this compound as active agent in nutraceutical and preventive novel therapies should be opened.

Introduction

Nowadays, *Helicobacter pylori* is one of the most important emerging human pathogens. Although the routes of transmission from the environment to humans are not completely defined, the entrance of *H. pylori* into the food chain has been identified as one of the dissemination pathways. [2] To date, DNA of *H. pylori* has been found mainly in water, raw milk, meat products and fresh salads, and the *H. pylori* prevalence values range from 2-30 % depending on the considered product. [3-5]

H. pylori invades the gastric mucosa and infects humans producing several digestive tract disorders, such as chronic active gastritis, peptic ulceration, and in severe cases, gastric cancer. This organism was definitively classified as the unique biological carcinogenic agent in 1994.[6] One of the most concerning points regarding the eradication of this pathogen is the critical resistance that *H. pylori* has developed in recent years against the current antimicrobial applied therapies (the effectiveness has decreased to 70 % compared to other antibiotic therapies against other infectious pathogens that are 95 % effective) highly concerning for the scientific community.[7]

Under the urgent claim of the World Health Organization in 2017 to find alternative antimicrobial strategies to fight against the most resistant human pathogens,[6] novel natural antimicrobials have been investigated. In that sense, several compounds of vegetable and animal origin have shown antimicrobial capability against *H. pylori*, some of which were used from ancient times to avoid gastrointestinal problems.[7] The effect of lactoferrin from bovine milk against *H. pylori* was studied by Di Mario et al., (2003)[10]. This compound showed a synergistic effect against *H. pylori* proliferation *in vivo* used in combination with antibiotics. Catechins from green tea, and quercetin glycosides from apple peel, have shown strong anti-urease activity affecting *H. pylori* membrane functionality. [11,12] Recently, propolis from the honeybee *Apis mellifera* has also been identified as an effective compound against this pathogen with a chemoprotective effect on gastric epithelial cells.[13] In addition, phenolic compounds have been suggested to have a great antimicrobial potential against *H. pylori*.[14,15] Additional studies regarding the mechanism of polyphenol action inhibiting *H. pylori* growth have indicated that under exposure to these antimicrobial phytochemicals, *H. pylori* enters in coccoid form, remaining unable to grow. Terpenes from essential oils and phenolic compounds from ginger have

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also been described as alternative natural antimicrobials against *H. pylori*.[16,17]

Since the last 10 years, novel advances regarding the influence of the gastrointestinal microbiome on *H. pylori* inhibition, and on individual and population-based gastric cancer prevention strategies, have been studied.[18] Compounds of marine origin have recently been used as ingredients with high applicability in the pharmacological, food and aesthetic industries.[19] Currently, algae constitutes a sustainable source of bioactive molecules, with brown algae from the *Phaeophyceae* group primarily rich in complex carbohydrates, with high prebiotic, immune-modulator and antioxidant potential.[22, 23] Among them, the complex fucoidan, sulphated polysaccharide of brown algae, has been described to significantly encourage the growth of beneficial intestinal microbiota, stimulates the immune system; and inhibit the viral replication; and has antioxidant, anti-inflammatory and anticancer properties,[24-26] hence fucoidan has been named as "*nutrient of the future*"[26]. According to the review of Morya et al., (2012),[27] since fucoidan was first isolated in 1918, more than eight hundred articles focusing on fucoidan have been cited in PUBMED. Although, fucoidan extracts from algae have not been approved for use in biomedical applications, research on the bioactivity of this compound has increased exponentially in recent years (e.g. as promising agents in drug delivery, biomaterials, topical agents, and orally delivered agents for a variety of pathologies)[28]. Fucoidan has the status of generally recognized as safe "*GRAS*" in the USA, Canada, Australia, and recently in Europe (approval December 2017).[29] However, to our knowledge only one previous publication has addressed the *in vivo* evaluation of fucoidan as a possible anti-*H. pylori* agent. [30] Recently, the nematode *Caenorhabditis elegans* has been incorporated as a whole animal screening platform for antimicrobials.[31-33] This organism has a rapid generation time (300 genetically identical progeny in a 3-day life cycle). The entire genome of this self-fertilizing hermaphrodite nematode has been sequenced. *Caenorhabditis elegans*, is an invertebrate animal model that has a high homology to the human genome and mimics human physiological responses.[34] Using this novel model, Moy et al., (2009)[35] tested more than 40,000 compounds and extracts, and identified 28 novel antimicrobials against *Enterococcus faecalis and Candida albicans*. In fact, many of the virulence factors involved in the killing of worms have been identified and also required for the pathogenesis of mammals.[36]

The aim of the present study is to evaluate the *in vitro* and *in vivo* antimicrobial potential of fucoidan against *H. pylori*, by testing the effectiveness of fucoidan from three different *Phaeophyceae* species, *Fucus vesiculosus, Macrocystis pyrifera*, and *Undaria pinnatifida*. The origin and concentration of fucoidan were evaluated in terms of bacteriostatic and bactericidal potential, and the protective effect of fucoidan against *H. pylori* proliferation was assessed *in vivo* using *Caenorhabditis elegans* model. The obtained results could contribute to the future development of promising human therapies anti-*H. pylori*.

Material and Methods

Helicobacter pylori bacterial culture

The strain of *H. pylori* used in the present study was provided by the United Kingdom Culture Type Collection with reference number 11637 NCTC. The lyophilized culture was revived according to the protocol provided by the NCTC. Cells were grown under optimal conditions (37 °C, microaerobic conditions: O_2 5 %; CO_2 15 %; N_2 85 %) in liquid Brucella Broth medium (BB) (B3051 Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) supplemented with 5 % (v/v) sterile foetal Bovine Serum (FBS) for 5-7 days until the stationary phase was reached.[37,38] Cells recovered by centrifugation were washed three times with 5 % BB-FBS; homogenized aliquots were placed into Eppendorf tubes (2 ml) and preserved at -80 °C until use. The final concentration of the culture stock was $1\pm0.54x10^8$ CFU/ml.

Fucoidan extracts

Fucoidan extracts included in the present study (purity≥95 %) were provided as powders by Merck KGaA International Company (Darmstadt, Germany) and included: *Fucus vesiculosus* (F8190 Sigma; molecular weight 82 kDa; sulfate content 24.5 % (w/w); most abundant monosaccharides: mannose (1.27 %), fucose (38 %), and galactose (3 %)); *Macrocystis pyrifera* (F8065 Sigma; molecular weight 176 kDa; sulfate content 27 % (w/w); most abundant monosaccharides: mannose (1.12 %), fucose (26 %), and galactose (4 %)); and *Undaria pinnatifida* (F8315 Sigma; molecular weight 51 kDa; sulfate content 30 % (w/w); most abundant monosaccharides: mannose (5 %), fucose (39 %), and galactose (27 %))[39].

Fucoidan antimicrobial suspensions

For each of the considered *Phaeophyceae* species, a stock solution at concentration of 5000 µg/mL was prepared in Müeller Hinton broth (MHB) (70192 Sigma-Aldrich; Merck KGaA International Company (Darmstadt, Germany)). For each of the independent trials, the suspensions were aliquoted in 2 ml Eppendorf tubes and stored at -20 °C for use.

Suspensions of fucoidan at concentrations of 5, 10, 25, 50 and 100 µg/ml were prepared in 10 ml tubes containing MHB+FBS (5 %) from the initial 5000 µg/ml stock. Liquid media MHB+FBS (5 % no-supplemented with fucoidan) was used as the control broth.

Inoculation and microbial analysis

H. pylori stock cells were revived under optimal conditions. Stocks of bacterial solution (100 µl) was spread on plates of Columbia blood agar (CBA, Difco, Franklin Lakes, New Jersey, USA) supplemented with defibrinated horse blood (10 %) (HB, Oxoid, UK) (CBA+HB 10 %) and incubated at 37 °C under microaerobic conditions. Seven-day-old cultures were harvested by scraping the bacterial growth with a sterile swab. Recovered cells were resuspended in both (i) MHB+FBS 5 % without fucoidan (considered as a control of bacterial growth) and; (ii) MHB+FBS 5 % with [5-100] µg/ml fucoidan

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suspensions. In both cases the initial optical density (OD) at 600 nm was fitted to 0.10±0.05.

The effect of fucoidan on the microbial growth / inhibition of *H. pylori* at 25 °C was registered by measuring $OD_{600\ nm}$ at regular time intervals, 12-24h) using a Biomate 3 (Thermo Scientific, S.A.) spectrophotometer. Additionally, for each time interval, 100 µl aliquots were also taken in duplicate from each suspension (supplemented / not supplemented with fucoidan). Serial 10-fold dilutions of all aliquots were prepared in sterile PBS solution (1X (130 mmol/l sodium chloride, 10 mmol/l sodium phosphate, pH 7.2)), and seeded on CBA+HB (10 cm) plates. Plates were incubated at 37 °C for 5-7 days under microaerobic conditions in anaerobic jars (Campy Gas Pak system; Oxoid, Basingstoke, UK) prior to bacterial counting. All suspensions prepared in the present study were replicated without bacterial inoculation, and were considered as the blank for the OD measurements. These suspensions were incubated at 25 °C, under microaerobic conditions, to assess possible background contamination during the process.

Caenorhabditis elegans strain and growth conditions

C. elegans strain N2 was obtained from the College of Biological Sciences, Minnesota University, USA. Agar plates containing Nematode Growth Medium (NGM) were used to maintain nematodes (25°C) that were fed on a bacterial lawn of *E. coli* OP50.[40]

NGM plates supplemented with fucoidan were prepared. The most effective fucoidan according to *in vitro* studies was selected for the *in vivo* validation assay, which included NGM plates prepared with fucoidan at concentrations ranging from [5-100] µg/ml. To validate the protective capability of fucoidan against the invasion and infection of *H. pylori* in nematodes, age-synchronized nematodes were distributed on NGM+fucoidan plates on a bacterial lawn of *H. pylori* (10^4 $\log_{10}$ cycles per plate). The lifespan and egg-laying of *C. elegans*, and the *H. pylori* concentration in the digestive tract of the nematode were evaluated in nematodes grown on NMG plates seeded with *H. pylori* (considered as control), and nematodes grown on NMG+fucoidan plates.

The worms were maintained at 25 °C during their life cycle (approximately three weeks) and were examined at 24 h intervals for lifespan studies (10 nematodes per plate, 5 repetitions). Nematodes were transferred every day to fresh plates prepared with NGM or NGM+fucoidan and seeded with *H. pylori* (10^4 $\log_{10}$ cycles per plate). Worms were considered dead when they did not move and did not respond to stimulation (contact with a platinum worm picker).

For fertility analysis, first-stage larvae (L1) were transferred to fresh bacterial lawns (*E. coli* OP50 reference; and *H. pylori* control) in NGM, individually (25 replicates in 5 individual repetitions) marking the start of the experiment (time $t = 0$). Nematodes were transferred every day to fresh plates for the whole lifecycle of the nematode. Egg-counting was carried out every 6 h (3 times per day). The same procedure was carried out for nematodes fed with *H. pylori* on NGM plates supplemented with fucoidan at different concentrations. The reproductive success (RS) was measured as the total number of viable eggs laid per day by nematodes in each cohort (125 individual evaluated nematodes for each scenario).

Additionally, the *H. pylori* concentration (CFU per nematode) in the digestive tract of nematodes was quantified by real time quantitative polymerase chain reaction qRT-PCR assay.

Helicobacter pylori quantification by real time - quantitative polymerase chain reaction (qRT-PCR) based on SYBR green I fluorescence

For quantification assays (concentration of *H. pylori* in the digestive tract during *C. elegans* feeding), 5 age-synchronized nematodes were placed on a plate (NGM medium or NGM+fucoidan medium); 10 plates per assay condition, and 5 repetitions per scenario were used. Nematodes were transferred every 24h to freshly prepared plated during their complete life cycle. Grown nematodes were recovered from plates at different intervals (0, 1, 3, 5, 8, 10, 12, and 15 days) and washed in drops containing 5 µl of 25 mM levamisole in M9 buffer (LM buffer) for paralysis and inhibition of pharyngeal pumping and expulsion.[41] Then LM buffer was used to wash the nematodes twice more. Afterwards, the washed nematodes were placed in a 1.5 ml Eppendorf tube containing 50 µl of PBS buffer with 1% Triton X-100 and mechanically disrupted using a motor pestle. Nucleic acids were extracted from worm lysates using the GeneJet™ Genomic DNA Purification Kit (Fermentas, Baden-Württemberg, Germany) following the mammalian tissue protocol, according to the manufacturer's instructions. *Helicobacter* DNA was detected using a LightCycler® 2.0 Instrument (Roche Applied Science, Spain) according to the optimized qRT-PCR approach developed by Pina-Pérez et al., (2018). [42]

Statistical analysis

The significance of fucoidan antimicrobial potential against *H. pylori* was assessed by evaluating studied variables through ANOVA. To determine which levels of each factor were significantly different ($p \leq 0.05$) a multiple range test (MRT) was applied, and the Fisher distribution (LSD) was used to check equality of variances. Statgraphics® Centurion XV software (Statpoint Inc., Virginia, USA) was used for all the statistical analyses carried out in the present study.

Results

In vitro antimicrobial potential of fucoidan from *Phaeophyceae* species against *H. pylori*

Figure 1 shows the kinetic behaviour of *H. pylori* at 25 °C, in the control reference liquid medium MHB+FBS (5 %) and in media supplemented with fucoidan at different concentrations. As seen graphically, the highest antimicrobial effects are shown for the high fucoidan concentrations applied, independent of the fucoidan origin. Moreover, under the same incubation conditions and concentrations applied,

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the origin of fucoidan significantly affected (p-value ≤0.05) the bactericidal/bacteriostatic effect exerted against *H. pylori*. Table 1 includes the fucoidan concentrations required to exert a bacteriostatic or bactericidal effect against *H. pylori* depending on the origin species.

Fucoidan [5-100] µg/ml from *Undaria pinnatifida* resulted in the most effective reduction in *H. pylori* bacterial counts (bacterial count reduction ≈ 2 to 4 $\log_{10}$ cycles). At this incubation temperature (25°C), fucoidan from *Macrocystis pyrifera* at concentrations in the range of [50-100] µg/ml effectively inhibited bacterial growth showing bacteriostatic effects. The values of the final bacterial load remained close to the initial bacterial load inoculated (≈4.25±0.077 $\log_{10}$ cycles). During the first 5 days of incubation, [50-100] µg/ml fucoidan from *Macrocystis pyrifera* effectively inhibited 2.30 $\log_{10}$ cycles of *H. pylori*. Additionally, cell exposure to 100 µg/ml fucoidan from *Macrocystis pyrifera* resulted in no viable culturable (VC) forms of *H. pylori* after 7 days of incubation. Two hypothesis can be proposed based on this result: (i) due to the stressful effect that fucoidan causes on *H. pylori* cells, the cells become in a coccoid form (viable but not culturable, VBNC), or (ii) due to the bactericidal effect of the fucoidan, cells become inactivated .

Fucoidan from *Fucus vesiculosus* presented a high antimicrobial effect against the *H. pylori* population, with the lowest concentrations from [5-10] µg/ml to control bacterial growth, exerting a significant bacteriostatic effect; 2.80±0.07 $\log_{10}$ cycles inhibition was observed with 10 µg/ml (7 days, 25 °C). The antimicrobial effect of fucoidan from *Fucus vesiculosus* was bactericidal at concentrations in the range of [25-100] µg/ml. The higher the concentration of fucoidan, the faster the microbial inactivation was (p-value ≤ 0.05); [50-100] µg/ml was able to reduce the bacterial load 1.60±0.15 and 2.60±0.24 $\log_{10}$ cycles, respectively, after just 24 h of microbial exposure. After 3 days of exposure to fucoidan from *Fucus vesiculosus*, 3.55±0.28 $\log_{10}$ cycles of microbial inactivation were achieved in suspensions containing 100 µg/ml fucoidan.

According to our results, fucoidan from brown algae *Undaria pinnatifida* was the most effective against *H. pylori* at 25°C. Taking into account the exposure of bacterial cells to fucoidan, after 24 h of incubation, concentration levels in the range of 50-100 µg/ml showed bactericidal effects. After 48 h of exposure, concentrations in the range of 25-50 µg/ml achieved a reduction of 2.30±0.25 $\log_{10}$ in the VC population of *H. pylori*. The concentration of 100 µg/ml was completely effective in reducing the *H. pylori* VC cells to below the detection limit (bactericidal effect = 4.10±0.12 $\log_{10}$ cycles). Even at low concentrations [10-25] µg/ml, fucoidan from *Undaria pinnatifida* showed bactericidal effects, being able to reduce the VC population of *H. pylori* from 4.85±0.12 to 6.85±0.24 $\log_{10}$ cycles with respect to the control, after 7 days of exposure at 25 °C.

An ANOVA analysis was performed to determine the significance of the studied factors in reducing the level of *H. pylori*. The fucoidan origin (p value ≤ 0.05); concentration, (p value ≤ 0.05); and exposure time (p value ≤ 0.01) were significant factors affecting the antimicrobial capability of this bioactive compound.

For all the studied conditions, and considering the three *Phaeophyceae* species included in the present research, it can be concluded that the higher the exposure time of bacterial cells to fucoidan, the higher the antimicrobial effect exerted by each of the considered fucoidan suspensions. Moreover, the higher the concentration of fucoidan present in liquid media, the higher the reduction of VC counts of *H. pylori*, [50-100] µg/ml of fucoidan was always effective and completely reduced (≈3.39-4.48 $\log_{10}$ cycles) the bacterial counts, in 2-7 days depending on fucoidan origin.

Caenorhabditis elegans as a model for *Helicobacter pylori* infection: validation of the protective effect of fucoidan

Fucoidan from *Undaria pinnatifida* was selected to test the *in vivo* antimicrobial potential of this compound using the *C. elegans* model. Figure 2 shows the survival function of *C. elegans* fed with *H. pylori* in NGM medium and NGM medium supplemented with fucoidan at different concentrations. As seen graphically, there was a significant increase in the survival capability of the nematode fed with *H. pylori* in the presence of fucoidan, even when this compound was added at the lowest concentrations (5 µg/ml). Meanwhile, the bactericidal potential of fucoidan was exerted *in vitro* in the concentration range of 50-100 µg/ml, in the *in vivo* assay, 5 µg/ml of fucoidan from *Undaria pinnatifida* was effective in increasing the lifespan of *C. elegans*, from 10 days in NGM (*C. elegans* fed with *H. pylori*) to 17 days in NGM + 5 µg/ml fucoidan. The addition of fucoidan at a concentration ≥25 µg/ml increased the lifespan of the nematode (26±2 days) even more than the lifespan observed in the reference (23 days lifespan when *C. elegans* in NGM was fed with optimal *E. coli* OP50).

Regarding the fertility assay, *C. elegans* N2 fed with *E. coli* OP50 in NGM (reference conditions) showed an RS equal to 155±23 eggs laid per day in the first 7 days, with egg-laying significantly reduced from day 5 of the life cycle. Under the *H. pylori* feeding pattern in NGM, the nematodes egg-laying was interrupted just after the first 36 h, and in many cases, worms retained eggs in bag (36 out of 125). The number of viable offspring was also reduced significantly in nematodes fed with *H. pylori* in relation to nematodes fed under reference conditions (*E. coli* OP50) (Table 2). Working with NGM supplemented with fucoidan at different concentrations, the reproductive timing of *C. elegans* was extended (5-12 days) and additionally, the number of laid eggs was increased, which was directly related to the concentration of fucoidan added to the media (see Table 2).

To establish a relationship between *C. elegans* infection (reduced lifespan and reduced egg laying) and *H. pylori* accumulation in the digestive tract, *H. pylori* was quantified across the lifespan of the nematode by qRT-PCR. Table 3 shows the quantitative values detected for *H. pylori* depending on considered scenarios. *C. elegans* fed with *H. pylori* seeded in NGM medium showed an increase in the intestinal load

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from < 10^2 *H. pylori* CFU/worm on day 0 (L4 stage) to 4 10^5 CFU/worm on day 7, and only 12 % of the initial population was able to survive. Nematodes grown on NGM+ fucoidan plates showed < 10^2 *H. pylori* CFU/worm under ≥ 10 µg/ml fucoidan exposure, with no significant differences between digestive colonization of *C. elegans* when fucoidan was added in the range 10-100 µg/ml.

Discussion

Fucoidans from *Phaeophyceae* have been described to be more effective as antitumoural agents than other fucoidans from other algae species, even more, fucoidans from *Phaeophyceae* have a wide spectrum of functionalities not attributed to other fucoidans.[23,24,43] The present study concludes the significant differences between the fucoidans from three species of *Phaeophyceae* evaluated for their antimicrobial capability against *H. pylori*. Although the fucoidans resulted in all cases effective exerting both bacteriostatic and bactericidal effects [44], fucoidan from *Undaria pinnatifida* was the most effective, with bactericidal potential even at the lowest concentrations [5-10] µg/ml, depending on the exposure time. Previous studies outlined different bioactivities associated with fucoidans derived from different species, and additional bioactive potentials for fucoidan fractions derived from the same species have been observed [26,45] depending on the molecular weight (high or low) of fucoidan.[46,47,48] The specific bioactivities exerted by fucoidan have been mainly associated to the structure of fucoidan molecules and fucose groups.[49,50] According to Mak et al., (2014)[49] the selective cytotoxicity of fucoidan against cancer cells has been related to the sulfate content, uronic acid content, and molecular weight of different fucoidan fractions [50]. However, scarce information has been published regarding the relationship that exists between fucoidan structure and the bioactivity of these molecules.

Specifically, the antimicrobial effects of sulfated polysaccharides against *H. pylori* have been described as multimodal actions (mainly, reinforcement of adaptative immunity cells and antioxidant effects). [51,52,53] The anti-*Helicobacter pylori* effect of sulfated polysaccharides was previously reviewed by Besednova et al. (2015). [51] According to Besednova et al. conclusions, sulfated polysaccharides from *Phaeophyceae* demonstrated *in vitro* anti-ulcer effects, prevention of the adhesion of *H. pylori* to gastric cells, and reduction in the *H. pylori* capability to from biofilms. Fucoidan concentrations in the range 100-1000 µg/ml are required *in vitro* to show anti-*H. pylori* significant effects. [52]

In the present study, the *in vitro* and *in vivo* concentration levels of fucoidan showing bactericidal potential against *H. pylori* were very low for *Undaria pinnatifida* (5-100 µg/ml). Previous studies by Chua et al., (2015)[52] revealed that fucoidan from *Undaria pinnatifida* reduced the adherence of *H. pylori* to human gastric adenocarcinoma epithelial cells (AGS) when added at a concentration of 100 µg/ml. Also, Palanisamy et al. (2017)[24] showed significant antimicrobial effects of fucoidan from *Spatoglossum asperum* at concentrations in the range 100-150 µg/ml. Furthermore, Lee et al., (2013)[54-] previously demonstrated the synergistic effect of fucoidan in combination with antibiotics, reducing oral pathogenic bacteria. However, no previous study detected a reduction in *H. pylori* proliferation in culture (bacteriostatic or bactericidal effect) during an exposure time of 24-48 h. In contrast, and according to the results obtained in the present study, it was demonstrated that the antimicrobial potential of fucoidan against *H. pylori* was significantly enhanced by both, the fucoidan concentration added to the medium, and the exposure time (0-7 days). According to the results of Mak et al., (2014)[50], the addition of 100 µg/ml fucoidan from *Undaria pinnatifida* to the medium dislodged *H. pylori* from host cell surface. Combining the results from both studies, it is possible to infer that under the correct dosage, novel drug development can be carried out, including fucoidan as complement to antibiotics in the treatment of *H. pylori* infection, with the effectiveness of the treatment optimized based on the ingested fucoidan concentration (anti-adherence / anti-proliferation / and killing effects on *H. pylori*), and also based to treatment exposure time. [50,51,52,54]

Bioactive compounds from algae have also been tested *in vivo* using the *C. elegans* model.[55,56,57] During its growth, the worm intakes nutrients from the medium, in addition to the ingestion of the bacterial food source (*E. coli* OP50 or *H. pylori* in this case). Methanolic extracts from red alga, *Chondrus crispus,* have been demonstrated to increase the *C. elegans* lifespan increasing the oxidative stress tolerance of the nematode.[56] Astaxanthin (AX) from marine origin has also been described with high impact, increasing the lifespan of *C. elegans* populations fed on medium supplemented with 0.1 to 1 mM AX by 16-30 % (*E. coli* OP50 as food source).[54] In the present study, under fucoidan intake, the ingestion of *H. pylori* was reduced and the resistance of the nematode to this pathogen was improved (longer lifespan, improved fertility rate). Under fucoidan ingestion, *C. elegans* recovered the capability to grow even in the presence of *H. pylori*, up to levels corresponding to the pattern of nematodes fed *E. coli* OP50. Similar results were detected for the fertility assays; increasing the RS was increased close to 5-fold due to the addition of 100 µg/ml fucoidan to the media addition to the media. Confirming the lower values of *H. pylori* present in the nematode fed under fucoidan exposure, it was assumed that there was a possible combined protective effect between the antioxidant and fucoidan by signaling specific defense pathways in the nematode (e.g avoiding the ingestion of *H. pylori*) and the effective antibacterial potential of this compound exerted on *H. pylori* cells (anti-adherence and bactericidal activity at the *in vivo* level).[53,54] According to Ewald (2018)[56], reactive oxygen species (ROS) and antioxidant intake homeostasis are important for extracellular matrix integrity, pathogen defense, oxidative stress resistance, and longevity in *C. elegans,* probably explaining the synergy between fucoidan effects (the direct antioxidant potential, pathogen defense and antimicrobial specific potential of this molecule were

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influenced/improved in the nematode,) preventing *H. pylori* infection.

Conclusions

Fucoidan from *Undaria pinnatifida* had the highest bacteriostatic and bactericidal potential against *H. pylori* even at low concentrations, in the range of [5-10] µg/ml. Exposure time was a determining factor reducing *H. pylori* to close to 4 log_{10} cycles just after 7 days of exposure at 25 °C. The *in vitro* antimicrobial potential of fucoidan from *Undaria pinnatifida* was confirmed in *C. elegans,* an *in vivo* model for infection. For *C. elegans* fed *H. pylori,* supplementation of the media with fucoidan (100 µg/ml) increased the *C. elegans* lifespan from 10 (NGM) to 26 days (NGM+fucoidan). These results open a new promising line of research regarding the development of nutraceutical ingredients derived from fucoidan as complementary therapies to be applied in *H. pylori* infection treatment. The beneficial effects associated to this algae ingredient are being extensively reported [2010-2020] and it is important to highlight the future application (food preservation, pharmaceutical, biotechnological, and medicinal) of this compound as a sustainable and effective alternative antimicrobial answering the demand of the WHO regarding the urgent need for agents against concerning antibiotic resistant pathogens.

Conflicts of interest

In accordance with our policy on Conflicts of interest please ensure that a conflicts of interest statement is included in your manuscript here. Please note that this statement is required for all submitted manuscripts. If no conflicts exist, please state that "There are no conflicts to declare".

Acknowledgements

The present research work has been supported by the funds provided by the Spanish Ministry of Economy and Competitiveness (MINECO) under both projects with reference AGL2014-53875-R and AGL2017-86840-C2-2-R. M.C. Pina-Pérez is also grateful to the EC providing a H2020 MSCA – IF Grant Agreement n° 748314.

Notes and references

1 EUROPEAN COMMISSION. Risk assessment of food borne bacterial pathogens: Quantitative methodology relevant for human exposure assessment. HEALTH & CONSUMER PROTECTION DIRECTORATE-GENERAL Directorate C Scientific Opinions C1 - Follow-up and dissemination of scientific opinions, 2002, 1–81.

2 C. Palomino and E. Tomé, *Helicobacter pylori*: Rol del agua y los alimentos en su transmisión. *An. Venez. Nutr.* 2012, **25(2)**, 93-85.

3 M. Fernández, M. Contreras, P. Suárez, P. Gueneau, M.A. García-Amado, Use of HP selective medium to detect *Helicobacter pylori* associated with other enteric bacteria in seawater and marine molluscs, *Lett. Appl. Microbiol.,* 2007, **45**, 213–218.

4 S. Mousavi, F.S. Dehkordi, E. Rahimi, Virulence factors and antibiotic resistance of *Helicobacter pylori* isolated from raw milk and unpasteurized dairy products, *JVATiTD*, 2014, **20(51)**, 1–7.

5 E. Yahaghi, F. Khamesipour, F. Mashayekhi, F. Safarpoor Dehkordi, M.H. Sakhaei, M. Masoudimanesh, M. Khameneie, *Helicobacter pylori* in vegetables and salads: genotyping and antimicrobial resistance properties, *BioMed Res. Int.*, 2014, 757941, 1-11.

6 IARC-WHO (International Agency for Research on Cancer-World Health Organitation). (2014). Helicobacter pylori Eradication as a Strategy for Preventing Gastric Cancer. Working Group Reports (Vol. 8) 190 pag. https://www.iarc.fr/en/publications/pdfsonline/wrk/wrk8/Helicobacter_pylori_Eradication.pdf. (Accessed April 2019).

7 S.Y. Kim, D.J. Choi, J.W. Chung, Antibiotic treatment for *Helicobacter pylori*: Is the end coming?, *World J. Gastrointest. Pharmacol. Ther., 2015,* **6(4)**, 183–98

8 WHO. WHO publishes list of bacteria for which new antibiotics are urgently needed. 27 February 2017 | GENEVA – WHO. 2017. http://www.who.int/mediacentre/news/releases/2017/bacteria-antibiotics-needed/en/. Accessed February 27, 2019.

9 H. Takeuchi, V.T. Trang, N. Morimoto, Y. Nishida, Y. Matsumura, T. Sugiura, Natural products and food components with anti-*Helicobacter pylori* activities, *World J. Gastroenterol.,* 2014, **20(27)**, 8971-8.

10 F. Di Mario, G. Aragona, N. Dal Bò, G.M. Cavestro, L. Cavallaro, V. Iori, G. Comparato, G. Leandro, A. Pilotto, A. Franzè, Use of bovine lactoferrin for *Helicobacter pylori* eradication, *Dig. Liver Dis.*, 2003, **35(10)**, 706–710.

11 F. Di Mario, L.G. Cavallaro, C. Scarpignato, Rescue therapies for the management of *Helicobacter pylori* infection, *Digestive Dis.*, 2006, **24(1-2)**, 113–130.

12 E. Pastene, H.N. Speisky, M. Troncoso, J. Alarcón, G. Figueroa, In vitro inhibitory effect of apple peel extract on the growth of *Helicobacter pylori* and respiratory burst induced on human neutrophils, *J. Agr. Food Chem.*, 2009, **57**, 7743–7749.

13 U. Shapla, M. Raihan, M. Islam, F. Alam, M. Solayman, S. Hua Gan, M. Hossen, M. Khalil, Propolis: The future therapy against *Helicobacter pylori*-mediated gastrointestinal diseases, *J. Applied Biomed.,* 2017, **16**, 81-99.

14 M. Matsushima, T. Suzuki, A. Masui, K. Kasai, T. Kouchi, A. Takagi, T. Shirai, T. Mine, Growth inhibitory action of cranberry on *Helicobacter pylori*, *J. Gastroenterol. Hepatol.*, 2008, **23(2)**, 175–180.

15 Y.T Lin, Y.I Kwon, R.G Labbe, K. Shetty, Inhibition of *Helicobacter pylori* and Associated Urease by Oregano and Cranberry Phytochemical Synergies, *Appl. Environ. Microbiol.,* 2005, **71(12)**, 8558–8564.

16 A. Nostro, L. Cellini, S. Di Bartolomeo, E. Di Campli, R. Grande, M.A Cannatelli, L. Marzio, V. Alonzo, Antibacterial effect of plant extracts against Helicobacter pylori, *Phytotherapy Res.*, 2005, **19**, 198–202.

17 T. Ohno, M. Kita, Y. Yamaoka, S. Imamura, T. Yamamoto, S. Mitsufuji, T. Kodama, K. Kashima, J. Imanishi, Antimicrobial activity of essential oils against *Helicobacter pylori*, *Helicobacter*, 2003, **8**, 207-215.

18 S. Moss, The Clinical Evidence Linking *Helicobacter pylori* to Gastric Cancer, *Cellular Mol. Gastroenterol. Hepatol.,* 2017, **3(2)**, 183–191.

19 M.C Pina-Pérez, A. Rivas, A. Martínez, D. Rodrigo, Antimicrobial potential of macro and microalgae against pathogenic and spoilage microorganisms in food, *Food Chem.,* 2017, **235**, 34-44.

 This journal is © The Royal Society of Chemistry 20xx

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20 N.V. Thomas, S.K. Kim, Beneficial Effects of Marine Algal Compounds in Cosmeceuticals, *Mar. Drugs,* 2013, **11(1)**, 146–164.

21 M. Wells, P. Potin, J. Craigie, J. Raven, S. Merchant, K. Helliwell, A. Smith, M. Camire, S. Brawley, Algae as nutritional and functional food sources: revisiting our understanding, *J. Appl. Phycol.,* 2017, **29(2)**, 949–982.

22 M. Hayes. B.k. Tiwari, Bioactive Carbohydrates and Peptides in Foods: An Overview of Sources, Downstream Processing Steps and Associated Bioactivities, *Int. J. Mol. Sci.,* 2015, **16(9)**, 22485–22508.

23 M. Alboofetileh, M. Rezaei, M. Tabarsa, M. Rittà, C. Donalisio, F. Mariatti, S. G. You, D. Lembo, G. Cravotto, Effect of different non-conventional extraction methods on the antibacterial and antiviral activity of fucoidans extracted from *Nizamuddinia zanardinii. Int. J. Biol. Macromol.,* 2019, **124**, 131-137.

24 S. Palanisamy, M. Vinosha, T. Marudhupandi, P. Rajasekar, N.M. Prabhu. In vitro antioxidant and antibacterial activity of sulfated polysaccharides isolated from *Spatoglossum asperum*. *Carbohydr. Polym.*, 2017, **170**, 296-304

25 Q. Shang, X. Shan, C. Cai, J. Hao, G. Li, G. Yu, Dietary fucoidan modulates the gut microbiota in mice by increasing the abundance of *Lactobacillus* and *Ruminococcaceae*, *Food Funct.,* 2016, **7**, 3224–3232.

26 J.H. Fitton, D.N. Stringer, S.S. Karpiniec, Therapies from Fucoidan: An Update, *Appl. Microbiol. Biotechnol.*, 2012, **93(1)**, 71–82.

27 V.K. Morya, J. Kim, E.K. Kim, Algal fucoidan: structural and size-dependent bioactivities and their perspectives, *Appl. Microbiol. Biotechnol.*, 2012, **93(1)**, 71–82.

28 Y.H. Lin, K.Y. Lu, C.L. Tseng, J.Y. Wu, C.H. Chen, F.L. Mi. Development of genipin-crosslinked fucoidan/chitosan/N-arginine nanogels for preventing *Helicobacter* infection. *Nanomed.*, 2017, **12(12)**, 1491-1510

29 Marinova Maritech® fucoidan. 2018. https://www.marinova.com.au/news/nutracos-article/. (Accessed April 2019).

30 H. Shibata, M. Iimuro, N. Uchiya, T. Kawamori, M. Nagaoka, S. Ueyama, S. Hashimoto, T. Yokokura, T. Sugimura, T. Wakabayashi. Preventive Effects of Cladosiphon Fucoidan Against *Helicobacter pylori* Infection in Mongolian gerbils. *Helicobacter,* 2003, 8(1), 59-65.

31 C. Kong, S.A. Eng, M.P. Lim, S. Nathan, Beyond Traditional Antimicrobials: A *Caenorhabditis elegans* Model for Discovery of Novel Anti-infectives, *Front. Microbiol.,* 2016, **7(1956)**, 1–14.

32 J.J. Ewbank, O. Zugasti, *C. elegans*: model host and tool for antimicrobial drug discovery, *Dis. Model Mech.,* 2011, **4(3)**, 300–304.

33 P.R. Hunt, The *C. elegans* model in toxicity testing, *J. Appl. Toxicol.,* 2017, **37(1),** 50–59.

34 Y. Gong, Y. Luo, J. Huang, J. Zhang, Y. Peng, Z. Liu, Z. Baoxue, Theanine improves stress resistance in *Caenorhabditis elegans*, *J. Funct. Foods*, 2012, **4**, 988–993.

35 T.I. Moy, A.L. Conery, J. Larkins-Ford, G. Wu, R. Mazitschek, G. Casadei, K. Lewis, A.E. Carpenter, F.M. Ausubel, High-throughput screen for novel antimicrobials using a whole animal infection model, *ACS Chem. Biol.*, 2009, **4**, 527–533.

36 M. Arvanitis, J. Glavis-Bloom, E. Mylonakis, *C. elegans* to anti-infective discovery, *Curr. Opinion Pharmacol.,* 2013, **13**, 769–774.

37 H. Yonezawa, T. Osaki, S. Kamiya, Biofilm Formation by *Helicobacter pylori* and Its Involvement for Antibiotic Resistance, *BioMed Res. Int.,* 2015, **914791**, 1-9.

38 M. Douraghi, Y. Talebkhan, H. Zeraati, F. Ebrahimzadeh, A. Nahvijoo, A. Morakabati, Multiple gene status in *Helicobacter pylori* strains and risk of gastric cancer development, *Digestion*, 2009, **80(3)**, 200-207.

39 W. Zhang, T. Oda, Q. Yu, J. Jin. Fucoidan from *Macrocystis pyrifera* has powerful immune-modulatory effects compared to three other fucoidans. *Mar. Drugs*, 2015, **13(3)**, 1084-1104.

40 M. Sanz-Puig, E. Lázaro, C. Armero, D. Alvares, A. Martínez, D.S. Rodrigo, Typhimurium virulence changes caused by exposure to different non-thermal preservation treatments using *C. elegans* , *J. Food Microbiol.*, 2017, **262**, 49-54.

41 C. Portal-Celhay, E.R. Bradley, M.J. Blaser, Control of intestinal bacterial proliferation in regulation of lifespan in *Caenorhabditis elegans, BMC Microbiol.*, 2012, **12(49)**, 1-17.

42 M.C. Pina-Pérez, A. González, Y. Moreno, M.A. Ferrús, *Helicobacter pylori* growth pattern in reference media and extracts from selected minimally processed vegetables, *Food Cont.,* 2018, **86**, 389-396.

43 R. Elizondo-González, L.E Cruz-Suárez, D. Ricque-Marie, E. Mendoza-Gamboa, C. Rodríguez-Padilla, L.M. Trejo-Ávila, In vitro characterization of the antiviral activity of fucoidan from *Cladosiphon okamuranus* against Newcastle Disease Virus, *Virol. J.*, 2012, **9**, 1–9.

44 S. Palanisamy, M. Vinosha, P. Rajasekar, R. Anjali, G. Sathiyaraj, T. Marudhupandi, S. Selvam, N.M. Prabhu, S. You. Antibacterial efficacy of a fucoidan frantion (Fu-F2) extracted from Sargassum polycystum. Int. *J. Biol. Macromol.*, 2019, **125**, 485-495.

45 G.C. Poveda-Castillo, D. Rodrigo, A. Martínez, M.C. Pina-Pérez, Bioactivity of Fucoidan as an Antimicrobial Agent in a New Functional Beverage, *Beverages*, 2018, **4(3)**, 64.

46 M. Tutor Ale, J.D Mikkelsen, A.S. Meyer, Important Determinants for Fucoidan Bioactivity: A Critical Review of Structure-Function Relations and Extraction Methods for Fucose-Containing Sulfated Polysaccharides from Brown Seaweeds, *Mar. Drugs*, 2011, **9(10)**, 2106–2130.

47 P. S. Saravana, Y.N. Cho, M. P. Patil, Y.-J. Cho, G.D. Kim, Y. B. Park, H. C. Woo, B.S. Chun. Hydrothermal degradation of seaweed polysaccharide: Characterization and biological activities. *Food Chem.,* 2018, **268**, 179-187

48 S.H. Yu, S.J. Wu, J.Y. Wu, D.Y. Wen, F.L. Mi. Preparation of fucoidan-shelled and genipin-crosslinked chitosan beads for antibacterial application. *Carbohydr. Polym.,* 2015, **126**, 97-107.

49 A.D Holtkamp, S. Kelly, R. Ulber, S. Lang, Fucoidans and fucoidanases—focus on techniques for molecular structure elucidation and modification of marine polysaccharides, *Appl. Microbiol. Biotechnol.,* 2009, **82**, 1–11.

50 W. Mak, S.K. Wang, T. Liu, N. Hamid, Y. Li, J. Lu, W. White, Anti-Proliferation Potential and Content of Fucoidan Extracted from Sporophyll of New Zealand *Undaria pinnatifida*, *Front. Nutr.*, 2014, 1–10.

51 N.M. Besednova, T.S. Zaporozhets, L.M. Somova, T.A. Kuznetsova. Review: prospects for the use of extracts and polysaccharides from marine algae to prevent and treat the diseases caused by *Helicobacter pylori. Helicobacter*, 2015, **20(2)**, 89-97.

52 E. G. Chua, P. Verbrugghe, T. T. Perkins, A. C. Y. Tay. Fucoidans Disrupt Adherence of *Helicobacter pylori* to AGS Cells In Vitro. *Ev. Bas. Complem. Altern. Med.,* 2015, 1-6.

53 M. Liu, Y.X. Liu, M.J. Cao, G.M. Lium Q.C. Chen, L.C. Sun, H.X. Chen. Antibacterial activity and mechanisms of depolynerized fucoidans isolated from *Laminaria japonica*. *Carbohydr. Polym.*, 2017, **172**, 294-305.

54 K.Y. Lee, M.R. Jeong, S.M. Choi, S.S. Na, J.D. Cha, Synergistic effect of fucoidan with antibiotics against oral pathogenic bacteria, *Arch. Oral Biol.,* 2013, **58(5)**, 482-492.

55 J.S. Sangha, D. Fan, A.H. Banskota, R. Stefanova, W. Khan, J. Hafting, J. Craigie, A.T. Critchley, B. Prithiviraj, Bioactive

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components of the edible strain of red alga, *Chondrus crispus*, enhance oxidative stress tolerance in *Caenorhabditis elegans*, *J. Funct. Foods*, 2013, **5**, 1180-1190.

56 S.A. Diaz, E.Q. Mooring, E.G. Rens, O. Restif, Association with pathogenic bacteria affects life-history traits and population growth in *Caenorhabditis elegans*, *Ecol. Evol.*, 2015, **5(8)**, 1653–1663.

57 C.Y. Ewald, Redox Signaling of NADPH Oxidases Regulates Oxidative Stress Responses, Immunity and Aging, *Antioxidants*, 2018, **7(10),** 130.

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